1 **Serotonin signals through postsynaptic $G\alpha_q$, Trio RhoGEF, and DAG to promote**
2 ***C. elegans* egg-laying circuit activity and behavior**

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6 60 pages with [6 Figures](#), [2 Tables](#) and [3 Movies](#)

Abstract

Activated G_{α_q} signals through Phospholipase-C β (PLC β) and Trio, a Rho GTPase exchange factor (RhoGEF), but how these distinct effector pathways promote cellular responses to neurotransmitters like serotonin remains poorly understood. We used the egg-laying behavior circuit of *C. elegans* to determine whether PLC β and Trio mediate serotonin and G_{α_q} signaling through independent or related biochemical pathways. Using genetic rescue experiments, we find that PLC β functions in neurons while Trio RhoGEF functions in both neurons and the postsynaptic vulval muscles. While G_{α_q} , PLC β , and Trio RhoGEF mutants all fail to lay eggs in response to serotonin, optogenetic stimulation of the serotonin releasing HSN command neurons restores egg laying only in PLC β mutants. PLC β mutants showed vulval muscle Ca^{2+} transients while strong G_{α_q} and Trio RhoGEF mutants had little or no vulval muscle Ca^{2+} activity. Treatment with Phorbol esters that mimic Diacylglycerol (DAG), a product of PIP $_2$ hydrolysis, rescued egg-laying circuit activity and behavior defects of G_{α_q} signaling mutants, suggesting both Phospholipase C and Rho signaling promote synaptic transmission and egg-laying via DAG production. DAG activates effectors including UNC-13, however, we find that phorbol esters, but not serotonin, stimulate egg laying in *unc-13* and PLC β mutants. These results support a model where serotonin signaling through G_{α_q} , PLC β , and UNC-13 promote neurotransmitter release, and that serotonin also signals through G_{α_q} , Trio RhoGEF, and an unidentified PMA-responsive effector to promote postsynaptic muscle excitability. Thus, the same neuromodulator serotonin can signal in distinct cells and effector pathways to coordinate activation of a motor behavior circuit.

28 Introduction

29 Neurons communicate in circuits via synaptic transmission to initiate, sustain, and
30 terminate behaviors. During neurotransmission, both synaptic vesicles and dense-core vesicles
31 fuse with the presynaptic membrane, releasing neurotransmitters and neuropeptides that
32 activate postsynaptic ion channels and G protein coupled receptors (GPCRs) (Betke et al., 2012;
33 Geppetti et al., 2015). While much has been learned about neurotransmitter signaling pathways
34 through ionotropic receptors, the diversity of GPCRs and their signaling pathways has
35 complicated our understanding of how their signaling exerts changes on cell excitability and
36 behavior. The G protein, $G\alpha_q$, is one of the major G proteins expressed in all excitable cells
37 (Offermanns, 2001; Simon et al., 1991; Wilkie et al., 1992). Activated $G\alpha_q$ signals through PIP_2 -
38 specific phospholipase- $C\beta$ ($PLC\beta$) to generate the second messengers, inositol 1,4,5
39 trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 activates the IP_3 receptor to release Ca^{2+} from
40 intracellular stores and activate downstream kinases, lipases, and ion channels (Berridge et al.,
41 2000; Huang, 1989; Li et al., 2014; Mujica and Gonzalez, 2011). The membrane lipid DAG has
42 been shown to recruit and activate numerous effector proteins including UNC-13 and Protein
43 Kinase C (Ananthanarayanan et al., 2003; Brose and Rosenmund, 2002; Lou et al., 2008;
44 Maruyama and Brenner, 1991; Rozengurt et al., 1997; Silinsky and Searl, 2003; Thore et al.,
45 2005), but whether these or other identified DAG targets function to transduce all forms of $G\alpha_q$
46 signaling *in vivo* remains an open question.

47 Genetic studies in the nematode worm *C. elegans* show that $G\alpha_q$ signaling through both
48 $PLC\beta$, and Trio promotes neurotransmitter and neuropeptide transmission. In *C. elegans unc-*
49 *73* gene encodes at least 8 isoforms of Trio, which has both Rac and Rho GTPase exchange
50 factor (GEF) DH/PH domains (Steven et al., 2005; Williams et al., 2007). *unc-73* mutations that
51 specifically affect the second Rho activating DH/PH GEF domain of Trio (Trio RhoGEF) disrupt
52 locomotion, feeding, and egg-laying behaviors (Williams et al., 2007), without causing the axon

53 pathfinding and neurodevelopmental defects observed in animals bearing *unc-73* Trio RacGEF
54 mutations that affect the first, Rac activating DH/PH GEF domain (Steven et al., 1998). In worms,
55 $G\alpha_q$ knockouts are lethal while PLC β or Trio RhoGEF single knockouts show defects in
56 neurotransmission that disrupt locomotion, feeding, egg-laying and other behaviors, resembling
57 $G\alpha_q$ loss-of-function mutants (Bastiani et al., 2003; Brundage et al., 1996; Hajdu-Cronin et al.,
58 1999; Lackner et al., 1999). Worms bearing mutations that disrupt both PLC β and Trio RhoGEF
59 phenocopy the larval arrest phenotype of $G\alpha_q$ null mutants, consistent with these two effectors
60 relaying most or all of the relevant $G\alpha_q$ signaling (Williams et al., 2007). Genetic and biochemical
61 studies showed that $G\alpha_q$ binding to and activation of the Trio RhoGEF domain to promote Rho
62 signaling is conserved in mammals (Chhatiwala et al., 2007; Rojas et al., 2007), however, it
63 remains unclear how PLC β and Rho signaling promotes neurotransmitter and neuropeptide
64 release *in vivo*. The larval lethality of $G\alpha_q$ null mutants can be rescued by the DAG-mimetic
65 phorbol ester, PMA (Reynolds et al., 2005), and PMA can also rescue the egg-laying defects of
66 PLC β , Trio RhoGEF double mutants (Williams et al., 2007). These results suggest $G\alpha_q$ signaling
67 through both PLC β and Trio may ultimately converge to regulate DAG levels and the activation
68 of downstream effectors. Both PLC β and Trio RhoGEF promote acetylcholine (ACh) release
69 from motor neurons that control locomotion (Lackner et al., 1999; Miller et al., 1999; Williams et
70 al., 2007), although mutations in Trio RhoGEF cause behavior defects more aligned with a
71 function in dense core vesicle release (Hu et al., 2011). PLC β is expressed in the nervous system
72 and intestine (Lackner et al., 1999), while $G\alpha_q$ and Trio show additional postsynaptic expression
73 in muscles (Bastiani et al., 2003; Lackner et al., 1999; Steven et al., 1998; Steven et al., 2005).
74 While re-expression of PLC β in motor neurons (Lackner et al., 1999) or Trio in all neurons
75 (Williams et al., 2007) rescue the locomotion behavior defects of their mutants, prior work has
76 shown that $G\alpha_q$ has additional functions in muscles (Bastiani et al., 2003) to promote egg laying

77 where Trio RhoGEF is also expressed (Steven et al., 2005). Indeed, loss of PLC β fails to
 78 suppress the hyperactive egg-laying phenotypes of G α_q gain-of-function mutants, consistent
 79 with G α_q signaling through other effectors like Trio RhoGEF to regulate egg laying (Bastiani et
 80 al., 2003). Indeed, *unc-73* RhoGEF mutations strongly suppress the hyperactive egg-laying
 81 behavior phenotypes of G α_o mutants unable to inhibit G α_q signaling (cite Williams et al. 2007).

82 Using genetics, optogenetics, pharmacology, and Ca²⁺ imaging techniques we have
 83 investigated how G α_q and its two effector pathways regulate egg-laying circuit activity and
 84 behavior. We find that PLC β functions in the presynaptic neurons while Trio RhoGEF signals in
 85 both neurons and muscles to promote egg laying. Loss of each of these effectors imparts specific
 86 defects in egg-laying behavior that indicate these proteins function in distinct cells to promote
 87 egg-laying circuit activity and behavior. Many of these defects can be rescued in part by
 88 treatment with phorbol esters that mimic DAG production. These results clarify that despite G α_q
 89 signaling through independent PLC β and Trio RhoGEF pathways, these effectors may ultimately
 90 converge to potentiate DAG levels which promote egg-laying behavior.

91 MATERIALS AND METHODS

92 Strains

93 *C. elegans* worms were maintained at 20°C on Nematode Growth Medium (NGM) agar plates
94 with *E. coli* OP50 as a source of food as described previously (Brenner, 1974). All behavior
95 assays and fluorescence imaging experiments were performed with age-matched adult
96 hermaphrodites aged 24-36 h after the late L4 stage. All strains used in this study are listed in
97 Table 1.

99 Molecular biology and transgenes

100 Vulval muscle GCaMP5 strains

101 Vulval muscle Ca²⁺ activity was recorded using GCaMP5G (Akerboom et al., 2013) which was
102 expressed along with mCherry from the *unc-103e* promoter (Collins and Koelle, 2013), as
103 previously described (Collins et al., 2016; Ravi et al., 2018a). The wild-type reporter strain,
104 LX1918 *vsIs164 [unc-103e::GCaMP5::unc-54 3'UTR + unc-103e::mCherry::unc-54 3'UTR + lin-*
105 *15(+)] lite-1(ce314) lin-15(n765ts) X* was used as described previously (Collins et al., 2016).
106 LX1918 males were crossed separately into DA823 *egl-30(ad805) I*, MT1434 *egl-30(n686) I*,
107 JT47 *egl-8(sa47) V*, MT1083 *egl-8(n488) V*, KG1278 *unc-73(ce362) I*, LX1226 *eat-16(tm761) I*,
108 CG21 *egl-30(tg26) I*, *him-5(e1490) V*, or KP1097 *dgk-1(nu62) X* hermaphrodites to generate
109 MIA140 *egl-30(ad805) I; vsIs164 lite-1(ce314) lin-15(n765ts) X*, MIA139 *egl-30(n686) I; vsIs164*
110 *lite-1(ce314) lin-15(n765ts) X*, MIA109 *egl-8(sa47) V; vsIs164 lite-1(ce314) lin-15(n765ts) X*,
111 MIA288 *egl-8(n488) V; vsIs164 lite-1(ce314) lin-15(n765ts) X*, MIA141 *unc-73(ce362) I; vsIs164*
112 *lite-1(ce314) lin-15(n765ts) X*, MIA287 *eat-16(tm761) I; vsIs164 lite-1(ce314) lin-15(n765ts) X*,
113 MIA286 *egl-30(tg26) I; vsIs164 lite-1(ce314) lin-15(n765ts) X*, and MIA296 *dgk-1(nu62) vsIs164*
114 *lite-1(ce314) lin-15(n765ts) X*, respectively. The corresponding gene mutation was confirmed by
115 phenotype, genotype, or both. Presence of *vsIs164* was confirmed observing the mCherry

marker, and *lite-1(ce314)* X was confirmed with PCR genotyping. Oligo sequences used for genotyping the corresponding mutations are shown in Table 2.

Trio RhoGEF-E transgenes

Pan-neuronal expression:

The *rab-3* promoter was used to drive expression of GFP alone or with Trio-RhoGEF-E. Briefly, plasmids KG#68 (*rab-3p::GFP*; 15 ng/μl) alone or with KG#281 (*rab-3p::unc-73e*; 50 ng/μL) were injected into KG1278 *unc-73(ce362)* I (Williams et al., 2007). For behavior experiments, five independent GFP-expressing transgenic lines were used, from which a single transgenic line from each was kept: MIA374 *unc-73(ce362)* I; *keyEx66* (expressing GFP alone) and MIA375 *unc-73(ce362)* I; *keyEx67* (expressing GFP and Trio RhoGEF-E). Plasmids KG#281(*rab-3p::unc-73e*) and KG#68(*rab-3p::GFP*) were kind gifts from Dr. Kenneth Miller.

Pan-muscle expression

Plasmid pKMC33 (*rgs-1p::mCherry*) was digested with NheI/KpnI and ligated with similarly digested pPD96.52 (Fire lab *C. elegans* Vector Kit 1999; 1608: L2534, Addgene) to generate pKMC166 (*myo-3p::mCherry*). Plasmid KG#281 (*rab-3p::unc-73e*) was digested with NheI and KpnI, and the insert was ligated into similarly digested pKMC166 to generate pPD3 (*myo-3p::unc-73e*). pKMC166 (15 ng/μL) alone or with pPD3 (50 ng/μL) was injected into KG1278 *unc-73(ce362)* I mutants. Five independent mCherry-expressing transgenic lines were used for behavior experiments from which a single transgenic line from each was kept: MIA376 *unc-73(ce362)* I; *keyEx68* (expressing mCherry alone) and MIA377 *unc-73(ce362)* I; *keyEx69* (expressing mCherry + Trio RhoGEF-E).

Neuron and muscle co-expression

Plasmids KG#68 (15 ng/μl; pan-neuronal GFP) or pKMC166 (15 ng/μl; pan-muscle mCherry) alone or with KG#281 (50ng/μl; pan-neuronal *unc-73e*) and pPD3 (50ng/μL; pan-muscle *unc-73e*) were injected into KG1278 *unc-73(ce362)* I, generating five independent mCherry(+), GFP(+) transgenic lines for behavior experiments from which a single transgenic line from each was kept: MIA372 *unc-73(ce362); keyEx64* expressing mCherry (muscles) and GFP (neurons) only and MIA373 *unc-73(ce362); keyEx65* expressing GFP (neurons), mCherry (muscles) and TrioRhoGEF-E (both neurons and muscles).

PLCβ Transgenes

To generate a control plasmid expressing GFP in neurons, GFP coding sequences were amplified from pJM60 (Moresco and Koelle, 2004) using oligonucleotides RE-GFP-FWD / -REV, digested with NheI / KpnI, and ligated into similarly digested pGP3 bearing the *rgs-1* promoter (Dong et al., 2000), generating pKMC78. An *egl-8* cDNA was used to generate and express a functional GFP fusion protein in neurons. Briefly, oligonucleotides *egl-8*-cDNA-fwd / -rev were used to amplified *egl-8* coding sequences from a plasmid bearing an *egl-8* cDNA provided by Dr. Kenneth Miller (pKP309). This amplicon was digested with NheI / NcoI and ligated into a similarly digested pPD49.26 plasmid, generating pKMC193. Quickchange mutagenesis with oligonucleotides *egl-8*-Cterm-NotI-fwd / -rev were used to insert an in-frame NotI site near the 3' end of the *egl-8* cDNA in a divergent region of the coding sequence, generating plasmid pKMC194. Coding sequences for *egl-8* bearing this NotI site were then moved to pKMC78 by digestion of pKMC194 with NheI/NcoI followed by ligation into a similarly digested pKMC78, generating pKMC195. Oligonucleotides NotI-GFP-FWD / -REV were used to amplified GFP coding sequences from pKMC78, digested with NotI, and ligated into a similarly digested pKMC195, generating pKMC196. A strain bearing the *egl-8(sa47)* mutation was generously provided by Dr. Joshua Kaplan and backcrossed four times to N2 wild-type animals to generate

166 LX1225 *egl-8(sa47)* V. MT8189 *lin-15(n765ts)* males were mated to LX1225 to generate LX1287
 167 *egl-8(sa47)* V; *lin-15(n765ts)* X hermaphrodites that were kept at 15°C prior to injection.
 168 Plasmids expressing GFP alone (pKMC78; 5 ng/μl) or *egl-8* CDNA fused to GFP (pKMC196; 5
 169 ng/μl) were injected along with pL15EK (50 ng/μl) into LX1287 hermaphrodites. For behavior
 170 experiments, five independent GFP-expressing lines were used from which a single transgenic
 171 line (*vsEx679* [GFP] and *vsEx680* [EGL-8::GFP], respectively) was kept. We noted that
 172 transgenic expression of GFP alone did cause a modest, but significant reduction of egg
 173 accumulation compared to LX1225 *egl-8(sa47)* V mutant animals. This effect appeared to be
 174 specific for egg accumulation, as these same *egl-8(sa47)* GFP-only expressing transgenic lines
 175 showed similar resistance to 1 mM aldicarb as LX1225 *egl-8(sa47)* V (0 ±2.1% vs. 9 ±5.9% of
 176 animals paralyzed at 4 h, respectively). In contrast, 48 ± 7.9% of transgenic *egl-8(sa47)* animals
 177 expressing EGL-8::GFP were paralyzed at 4 h, not significantly different to wild-type N2 animals
 178 (51 ±9.6% of animals paralyzed at 4 h).

179

180 **Vulval muscle Channelrhodopsin-2 strains**

181 N2 males were crossed into MIA229 *keyls48 [ceh-24p::ChR2::unc-54 3'UTR + lin-15(+)]*, *lite-*
 182 *1(ce314)*, *lin-15(n765ts)* X ([Kopchock et al., 2021](#)) to produce F1 heterozygous males, which
 183 then were crossed separately into MIA211 *unc-73(ce362)* I; *lite-1(ce314)* *lin-15(n765ts)* X,
 184 MIA299 *egl-30(ad805)* I; *lite-1(ce314)* *lin-15(n765ts)* X, MIA303 *egl-8(n488)* V; *lite-1(ce314)* *lin-*
 185 *15(n765ts)* X, or MIA307 *egl-8(sa47)* V; *lite-1(ce314)* *lin-15(n765ts)* X hermaphrodites to
 186 generate vulval muscle specific Channelrhodopsin-2 (ChR2) expressing transgenic lines
 187 MIA248 *unc-73(ce362)* I; *keyls48*; *lite-1(ce314)* X, MIA301 *egl-30(ad805)* I; *keyls48*; *lite-*
 188 *1(ce314)* X, MIA305 *egl-8(n488)* V; *keyls48*; *lite-1(ce314)* X, and MIA309 *egl-8(sa47)* V;
 189 *keyls48*; *lite-1(ce314)* X. The presence of *lite-1(ce362)* was confirmed by genotyping as above,

and the presence of the ChR2 transgene was confirmed by rescue of the *lin-15(n765ts)* multi-vulva (Muv) phenotype.

HSN Channelrhodopsin strains

ChR2 was expressed in the HSNs from the *egl-6* promoter via an integrated *wzIs30* transgene (Emtage et al., 2012). This transgene was crossed into $G\alpha_q$ signaling mutants as follows. N2 males were crossed into LX1836 *wzIs30 IV; lite-1(ce314) lin-15(n765ts) X* to generate heterozygous F1 males, which were then crossed separately into MIA211 *unc-73(ce362) I; lite-1(ce314) lin-15(n765ts) X*, MIA299 *egl-30(ad805) I; lite-1(ce314) lin-15(n765ts) X*, MIA303 *egl-8(n488) V; lite-1(ce314) lin-15(n765ts) X*, or MIA307 *egl-8(sa47) V; lite-1(ce314) lin-15(n765ts) X* hermaphrodites to generate MIA247 *unc-73(ce362) I; wzIs30 IV; lite-1(ce314) X*, MIA300 *egl-30(ad805) I; wzIs30 IV; lite-1(ce314) X*, MIA304 *wzIs30 IV; egl-8(n488)V; lite-1(ce314) X*, and MIA308 *wzIs30 IV; egl-8(sa47) V; lite-1(ce314) X*. The presence of *lite-1(ce362)* was confirmed by PCR genotyping, and the *wzIs30* transgene was confirmed by rescue of the *lin-15(n765ts)* Muv phenotype.

Behavior assays

Quantification of egg accumulation was performed as described (Chase and Koelle, 2004). Staged adults were obtained by picking late L4 animals and culturing them 24-30 h at 20°C. Each animal was placed in 7 μ L of 20% hypochlorite (bleach) solution and eggs were counted after animals had dissolved. Numbers of eggs and any internally hatched L1 animals were combined.

Pharmacological assays

Egg laying in response to exogenous serotonin was performed as described (Banerjee et al., 2017; Kopchock et al., 2021). Individual staged adult animals were placed in 100 μ l of either M9 buffer alone, or M9 containing 18.5 mM serotonin (creatinine sulfate monohydrate salt, Sigma-Aldrich #H7752) or M9 containing 10 μ M PMA (Phorbol-12-myristate-13-acetate, Calbiochem #524400) in a 96-well microtiter dish. After 1 hour, the number of released eggs and L1 larvae in each well were counted. Since egg-laying defective animals sometimes release one or two eggs/L1 larvae in response to mechanical stimulation when they are first picked into the well, animals were only recorded as responding if they laid 3 or more progeny. For calcium imaging, NGM plates containing either PMA or ethanol solvent were prepared as described (Reynolds et al., 2005). Age-matched adult worms from each genotype were placed on separate PMA or control NGM plates at room temperature for 1.5 hour. An agar chunk was then placed between two glass coverslips for Ca^{2+} activity recording as described (Ravi et al., 2018b). The unused plates were kept at 4 $^{\circ}\text{C}$ for future use.

Optogenetic assay

All-*trans* retinal (Sigma Aldrich, R2500) was resuspended in ethanol (100%) to make 100 mM solution and added to a warmed culture of OP50 bacteria grown in B Broth media to a final concentration of 0.4 mM. Individual NGM agar plates were seeded with 200 μ l of freshly prepared +ATR food and were grown in the dark for ~24 hours prior to use. In all photo-stimulation experiments, control animals were grown in the absence of ATR. Animals were imaged at 4x magnification on a Leica M165FC stereomicroscope and illuminated with 3.3 mW/cm² of $\sim 470 \pm 20$ nm blue light produced using a EL6000 metal halide light source and a GFP excitation/emission filter set. The 30 s on/off sequence was programmed and controlled using a Doric Optogenetics TTL Pulse Generator (OTPG-4, Version 3.3) triggering a SHB1 series shutter controller (ThorLabs; 170712-1).

239

240 **Microscopy**

241 **Ratiometric Ca²⁺ imaging**

242 Vulval muscle Ca²⁺ activity was performed in freely behaving adult animals at 24-30 h past the
 243 late L4 larval stage, as described previously ([Collins and Koelle 2013](#); [Collins et al. 2016](#); [Ravi](#)
 244 [et al. 2018a](#)). Worms co-expressing GCaMP5G and mCherry under the *unc-103e* promoter
 245 transgene *vs/s164* were mounted beneath the chunk of agar over the glass slide. Ca²⁺ activity
 246 was recorded through an 20X Apochromatic objective (0.8 NA) mounted on an inverted Zeiss
 247 Axio Observer.Z1. A Colibri.2 LED illumination system was used to excite GCaMP5 at 470 nm
 248 and mCherry at 590 nm for 10 msec every 50 msec. GFP and mCherry fluorescence emission
 249 channels were separated using a Hamamatsu W-VIEW Gemini image splitter and recorded
 250 simultaneously for 10 min with an ORCA-Flash 4.0 V2 sCMOS camera at 256 / 256-pixel
 251 resolution (4x4 binning) at 16-bit depth. The motorized stage was manually controlled using a
 252 joystick to maintain the freely behaving animal in the field of view. Image sequences were
 253 exported to Volocity software (Quorum Technologies Inc.) for segmentation and ratiometric
 254 analysis. Ca²⁺ transient peaks from ratio traces were detected using a custom MATLAB script,
 255 as described ([Ravi et al. 2018a](#)).

256

257 **Experimental design and statistical analysis**

258 Sample sizes for behavioral assays followed previous studies ([Chase and Koelle, 2004](#); [Collins](#)
 259 [et al., 2016](#)). Statistical analysis was performed using Prism v.8 or v.9 (GraphPad). Ca²⁺
 260 transient peak amplitudes, widths, and inter-transient intervals were pooled from multiple
 261 animals (typically ≥ 10 animals per genotype). All statistical tests were corrected for multiple
 262 comparisons (Bonferroni for one-way ANOVA or Fisher's exact tests; Dunn's correction for

263 Kruskal–Wallis tests). Each figure legend indicates individual p-values with $p < 0.05$ being
264 considered significant.

265

266

Results

Trio RhoGEF acts both in neurons and muscle to drive egg-laying behavior

Prior work has shown that $G\alpha_q$ signaling (Fig. 1A) through PLC β and Trio RhoGEF promotes neurotransmitter release and locomotion (Brundage et al., 1996; Miller et al., 1999; Williams et al., 2007). How $G\alpha_q$ signaling regulates other *C. elegans* behavior circuits is less well established. To address this uncertainty, we chose to examine the neural circuit driving egg-laying behavior (Fig. 1B). Egg-laying behavior in *C. elegans* is regulated by a small motor circuit with defined neurons and muscle connectivity (Cook et al., 2019; White et al., 1986). The HSNs are serotonergic command motor neurons (Fig. 1B) that initiate the egg-laying active state and promote the excitability of the vm1 and vm2 egg-laying vulval muscles (Collins et al., 2016; Emtage et al., 2012; Waggoner et al., 1998). Innervating ventral cord neurons release acetylcholine to regulate muscle contraction (Bany et al., 2003; Kim et al., 2001; Kopchock et al., 2021; Waggoner et al., 2000). We first examined the steady-state accumulation of eggs in the uterus as a proxy for changes in egg-laying circuit activity and behavior. As previously shown, animals bearing mutations in the EAT-16 RGS protein, which inhibits $G\alpha_q$ signaling (Hajdu-Cronin et al., 1999), or gain-of-function mutations in $G\alpha_q$ itself, (Bastiani et al., 2003; Doi and Iwasaki, 2002) showed a significant increase in egg laying resulting in a significant reduction in egg accumulation compared to the $\sim 12 \pm 3$ embryos retained in wild-type animals (Fig. 1C-E; Fig. 1I). Conversely, animals bearing mutations which reduce $G\alpha_q$ signaling showed the opposite phenotype. Animals bearing an early nonsense mutation predicted to be a PLC β null mutant, *egl-8(sa47)*, (Lackner et al., 1999; Williams et al., 2007) accumulated an average of 21 eggs, a significant increase (Fig. 1G). Animals bearing a missense mutation in the RhoGEF domain of Trio, *unc-73(ce362)* (Williams et al., 2007), showed an even stronger increase in egg accumulation, accumulating 31 eggs and closely resembling animals bearing loss-of-function

mutations in $G\alpha_q$ itself (Fig. 1F-1I). Together, these results confirm that $G\alpha_q$ and its effectors PLC β and Trio RhoGEF are required for egg-laying behavior in *C. elegans* and that loss of the Trio RhoGEF branch causes a stronger behavior impairment compared to loss of PLC β .

Previous work has shown that PLC β is expressed in neurons and in the intestine while $G\alpha_q$ and Trio are expressed in nearly all excitable cells including neurons and muscles (Bastiani et al., 2003; Brundage et al., 1996; Lackner et al., 1999; Miller et al., 1999; Steven et al., 1998).

To understand where $G\alpha_q$ and its effectors function to regulate egg laying, we used tissue-specific promoters to express cDNAs encoding PLC β or Trio RhoGEF in either all neurons, in the body wall and egg-laying vulval muscles, or in both neurons and muscles. We found that transgenic expression of PLC β from a pan-neuronal promoter in PLC β null mutants was sufficient to rescue their defects in egg laying (Fig. 1J) and acetylcholine (ACh) release as measured by restoration of sensitivity to aldicarb, a cholinesterase inhibitor (data not shown).

Previous work has indicated the presence of eight transcript variants of Trio (A, B, C1, C2, D1, D2, E, and F), which are differentially expressed in *C. elegans* (Steven et al., 1998; Steven et al., 2005). Transgenic expression of Trio RhoGEF-E in neurons is sufficient to rescue the locomotion defects of Trio RhoGEF mutants (Williams et al., 2007). To explore whether Trio RhoGEF acts similarly in neurons to promote egg laying, we used a pan-neuronal promoter to express Trio RhoGEF-E and measured egg accumulation in these animals. We observed a partial, but significant reduction in the number of eggs retained in Trio RhoGEF mutants (~36 eggs) compared to control Trio RhoGEF mutant animals (~42 eggs; Fig. 1K). In contrast, transgenic expression of Trio RhoGEF-E in the egg-laying vulval muscles from a muscle-specific promoter showed a much greater rescue of egg accumulation (~25 eggs), and this rescue of egg laying was improved to nearly wild-type levels when Trio RhoGEF was expressed in both neurons and muscles (~19 eggs; Fig. 1K). Together, these results confirm that $G\alpha_q$ effectors PLC β and Trio RhoGEF function in neurons to regulate egg-laying behavior. Our results also

suggest Trio RhoGEF also functions in the postsynaptic vulval muscles for proper regulation of egg laying, a finding consistent with previously results regarding $G\alpha_q$ (Bastiani et al., 2003).

Serotonin signals through $G\alpha_q$, Trio, and PLC β to promote egg laying.

Previous studies have shown that serotonin released from the HSNs signals through G-protein coupled serotonin receptors expressed on the vulval muscles (Bastiani et al., 2003; Dempsey et al., 2005; Fernandez et al., 2020; Tanis et al., 2008; Xiao et al., 2006). The vulval muscles are also innervated by cholinergic ventral cord motor neurons (Cook et al., 2019; White et al., 1986) whose release of ACh is regulated by serotonin and G protein signaling (Nurrish et al., 1999). To test whether serotonin promotes egg laying via activating $G\alpha_q$ signaling, we measured the egg-laying response to serotonin in $G\alpha_q$ signaling mutants. Serotonin promotes egg laying in hypertonic M9 buffer, a condition that normally inhibits egg laying in both wild-type and HSNs-deficient *egl-1(dm)* mutants, which developmentally lack the HSNs (Fig. 2B). Consistent with previous results (Bastiani et al., 2003; Brundage et al., 1996; Trent et al., 1983), nearly 78% of wild-type and 70% of HSN-deficient *egl-1(dm)* animals laid eggs in response to serotonin compared to only 13% of $G\alpha_q$ mutant animals (Fig. 2B). Serotonin response was similarly and significantly reduced to 23% and 3% in PLC β and Trio RhoGEF mutant animals, respectively (Fig. 2B). Our results are consistent with the previous results reporting that $G\alpha_q$ loss-of-function mutants and PLC β deletion null mutants, *egl-8(n488)* do not lay eggs in response to exogenous serotonin (Bastiani et al., 2003; Trent et al., 1983). However, we find that a second PLC β null mutant *egl-8(sa47)* similarly fails to lay eggs in response to exogenous serotonin, a result different from that previously reported (Bastiani et al., 2003). This discrepancy may arise from somewhat different concentrations of serotonin used. The distinct responses of animals without HSNs and animals lacking $G\alpha_q$ signaling indicate that $G\alpha_q$, PLC β and Trio act at least in part

outside of HSNs to promote egg laying in response to serotonin. To determine where Trio RhoGEF deficiency caused serotonin insensitivity, we measured egg laying in Trio RhoGEF mutant animals re-expressing Trio RhoGEF-E in either neurons, muscles, or both. Transgenic expression of Trio RhoGEF-E in neurons failed to rescue egg laying in response to serotonin (Fig. 2C), but expression of Trio RhoGEF-E in muscles, or in both neurons and muscles, restored egg laying of *unc-73(ce362)* mutant animals (Fig. 2C), suggesting Trio RhoGEF mediates serotonin signaling in the vulval muscles. Together, these results indicate that $G\alpha_q$, PLC β , and Trio RhoGEF function at least in part outside of the HSNs to drive egg laying in response to serotonin.

Optogenetic stimulation of the HSNs and vulval muscles suggests cellular specificity of $G\alpha_q$ effectors for egg-laying.

Optogenetic stimulation of Channelrhodopsin-2 (ChR2) expressed in either the HSNs (Emtage et al., 2012) or vulval muscles (Kopchok et al., 2021) can drive egg laying. To test whether and how $G\alpha_q$ and its effectors mediate this response, we expressed ChR2 in HSNs in $G\alpha_q$ and effector mutants and measured egg laying during 30s of exposure to blue light. Blue light stimulation of the HSNs drove an average release of ~3 eggs in wild-type animals, which was reduced to essentially zero in $G\alpha_q$ [*egl-30(ad805)*] and Trio RhoGEF [*unc-73(ce362)*] mutants (Fig. 3A), consistent to our previous results showing Trio RhoGEF acts in part in the postsynaptic vulval muscles downstream of the HSNs. In contrast, optogenetic stimulation of the HSNs in PLC β null mutants [*egl-8(sa47)* or *egl-8(n488)*] drove robust egg release, releasing an average of ~4 and ~6 embryos respectively in 30 s (Fig. 3A). The efficient rescue of egg laying in PLC β but not $G\alpha_q$ or Trio RhoGEF mutants upon optogenetic stimulation of HSNs is again consistent with distinct $G\alpha_q$, PLC β , and Trio RhoGEF requirements in the cells of the egg-laying circuit. To

test whether the failure of egg laying in $G\alpha_q$ and Trio RhoGEF mutants was a consequence of muscle developmental defects, rather than excitability deficits, we expressed and stimulated ChR2 in the vulval muscles. Blue light exposure drove the release of ~4 eggs in 30 s in wild-type animals. Both PLC β [*egl-8(sa47)* and *egl-8(nu488)*] and Trio RhoGEF mutants [*unc-73(ce362)*] laid a similar number of eggs as wild-type control animals after blue light stimulation (Fig. 3B). The *egl-30(ad805)* $G\alpha_q$ mutant laid slightly fewer eggs on average (~3) but this was not significantly different than wild type. Thus, the failure of $G\alpha_q$ and Trio mutants to lay eggs in response to exogenous serotonin or optogenetic stimulation of the HSNs does not arise from some intrinsic defect in vulval muscle contractility, but rather a specific deficiency in muscle excitability. These results are also consistent with prior findings showing rescue of egg-laying behavior defects of $G\alpha_q$, PLC β , and Trio RhoGEF mutants by exogenous phorbol esters (Lackner et al., 1999; Williams et al., 2007). Together, these results support the conclusion from our rescue experiments (Fig. 1J-1K) that PLC β and Trio RhoGEF function in distinct cells and through unique mechanisms to promote egg-laying circuit activity and behavior.

$G\alpha_q$ and Trio RhoGEF are required for vulval muscle Ca^{2+} activity

Egg laying in *C. elegans* is a two-state behavior where ~20 minute inactive states are punctuated by ~2-minute active states with high levels of Ca^{2+} activity in the egg-laying circuit driving release of 3-5 eggs (Collins et al., 2016; Waggoner et al., 1998; Zhang et al., 2008; Zhang et al., 2010). Loss of $G\alpha_q$ signaling in *egl-30(n686)* animals causes a significant reduction in spontaneous and serotonin-induced vulval muscle Ca^{2+} transients in immobilized animals (Shyn et al., 2003). We therefore tested whether these $G\alpha_q$ dependent Ca^{2+} activity defects were shared in PLC β and Trio RhoGEF mutants. We expressed the genetically encoded Ca^{2+} reporter, GCaMP5, along with mCherry in the vulval muscles of animals with either increased or decreased $G\alpha_q$ signaling

and performed ratiometric imaging in freely behaving animals. As shown in [Figure 4A](#), the normal two-state pattern of vulval muscle Ca^{2+} activity is lost in animals bearing strong loss-of-function $\text{G}\alpha_q$ or Trio RhoGEF mutations. In both the *egl-30(ad805)* $\text{G}\alpha_q$ and *unc-73(ce362)* Trio mutants, we failed to observe either the strong egg-laying Ca^{2+} transients where both the vm1 and vm2 muscles contract or even the smaller amplitude rhythmic ‘twitch’ Ca^{2+} transients ([Fig. 4A](#)) that are localized to the vm1 muscles ([Collins and Koelle, 2013](#)), strongly reducing the observed Ca^{2+} transient frequency ([Fig. 4B](#)). In contrast, the weaker *egl-30(n686)* $\text{G}\alpha_q$ mutant and two different PLC β null mutants showed grossly normal vulval muscle Ca^{2+} activity during both egg-laying and twitch transients ([Fig. 4A-C](#)). The elevated pattern of vulval muscle Ca^{2+} activity we observed in weak *egl-30(n686)* $\text{G}\alpha_q$ and PLC β mutants resembled *egl-1(dm)* animals lacking the HSNs ([Collins et al., 2016](#)). These animals still enter and leave infrequent egg-laying active states, possibly driven by the accumulation of eggs in the uterus ([Ravi et al., 2018a](#)). DAG Kinase- θ (DGK- θ) is thought to reduce DAG signaling through conversion to phosphatidic acid ([Fig. 1A](#)). Loss of the DGK- θ /DGK-1 increases neurotransmitter release and egg laying, likely through elevation of DAG levels and activation of downstream effectors ([Jose and Koelle, 2005](#); [Miller et al., 1996](#); [Nurrish et al., 1999](#)). To test if *dgk-1* mutants have increased egg-laying muscle activity, we performed Ca^{2+} imaging in *dgk-1(nu62)* mutants. Somewhat surprisingly, DAG Kinase mutant animals did not show a significant increase in vulval muscle Ca^{2+} activity ([Fig. 4A-C](#)). Like PLC β , DGK-1 is expressed in neurons ([Nurrish et al., 1999](#)), suggesting alterations of IP $_3$ and /or DAG levels in neurons may affect the frequency of egg-laying active states without altering the overall pattern or strength of vulval muscle Ca^{2+} activity within those active states. Indeed, *goa-1(n1134)* mutants that reduce inhibitory $\text{G}\alpha_o$ signaling have hyperactive egg-laying behavior defects that strongly resemble *dgk-1(nu62)* mutants without causing a correspondingly significant increase in vulval muscle Ca^{2+} activity ([Ravi et al., 2021](#)).

Conversely, animals with increased $G\alpha_q$ signaling either from a gain-of-function *egl-30(tg26)* mutations or *eat-16(tm761)* null mutations showed a significant increase in the amplitude of vulval muscle Ca^{2+} transients (Fig. 4A and 4C). We also observed a significant increase in the frequency of vulval muscle Ca^{2+} transients in *egl-30(tg26)* mutants, but this was not shared in *eat-16(tm761)* mutants. Together, these results indicate $G\alpha_q$ and Trio RhoGEF, but not PLC β , promote vulval muscle activity that drives twitching and egg-laying Ca^{2+} transients during egg-laying active states.

DAG mimetics restore muscle activity and egg laying to $G\alpha_q$ signaling mutants

How does serotonin signaling through $G\alpha_q$ promote vulval muscle activity? Previous results have shown that DAG mimetic phorbol esters restore locomotion and animal viability to $G\alpha_q$ null mutants and restore egg laying in PLC β , Trio RhoGEF double mutants (Williams et al., 2007). However, the effects of phorbol esters on egg laying in single mutants was not clear, raising questions as to whether PMA rescued egg laying downstream of PLC β , Trio RhoGEF, or both (Fig. 5A). We measured the egg-laying responses of wild-type animals and $G\alpha_q$ signaling mutants to Phorbol 12-myristate 13-acetate (PMA). As shown in Figure 5B, PMA treatment strongly stimulated egg laying in wild-type animals and all mutants with reduced $G\alpha_q$ signaling ($\geq 80\%$ animals laying eggs). Like serotonin (Fig. 2B), PMA also rescued egg laying in *egl-1(dm)* mutant animals lacking the HSNs (Fig. 4B), but only PMA rescued egg laying in $G\alpha_q$ and effector signaling mutants. These results are consistent with PMA acting downstream of both serotonin release from the HSNs and its subsequent signaling through $G\alpha_q$. We next imaged how exogenous PMA affected vulval muscle Ca^{2+} transient activity. We performed 10-minute GCaMP5 Ca^{2+} recordings of wild-type or $G\alpha_q$ signaling mutants treated for two hours with 10 μ M PMA (Movies 1-3). Quantitation of Ca^{2+} transients showed that PMA significantly increased

vulval muscle Ca^{2+} activity in wild-type animals to 10 ± 1.6 transients per minute from an average 2 ± 0.9 transients per min in vehicle-treated, wild-type animals (Fig. 5C-D and Movie 1). PMA also restored both rhythmic twitch and egg-laying Ca^{2+} transients to strong $\text{G}\alpha_q$ and Trio RhoGEF signaling mutants, with Ca^{2+} transients frequencies increasing from essentially 0 ± 0.8 transients per minute in vehicle-treated controls to 7 ± 2 transients per minute after PMA treatment, almost but not quite to the level of PMA-treated wild-type control animals (Fig. 5C-D and Movies 2-3). Together, these studies show that DAG-mimetic phorbol esters restore muscle excitability and contractility defects of $\text{G}\alpha_q$ and Trio RhoGEF mutants, suggesting DAG production is a major and necessary consequence of both $\text{G}\alpha_q$ and Trio RhoGEF signaling.

DAG promotes egg laying independent of UNC-13 and Protein Kinase C

How do phorbol esters such as PMA rescue vulval muscle Ca^{2+} activity and egg laying? Previous results have shown that DAG and PMA bind to C1 domain-containing proteins such as mUNC-13/UNC-13 and Protein Kinase C (PKC) to regulate their activity (Betz et al., 1998; Huang, 1989; Konig et al., 1985; Newton, 2001; Silinsky and Searl, 2003). To test if DAG regulates egg-laying behavior through activation of UNC-13 or PKC, we tested how PMA and serotonin affect egg laying in mutants lacking these proteins (Fig. 6). Mutants that eliminate axonal and synaptic UNC-13 show reduced egg laying, accumulating an average of 22 eggs compared to 15 seen in wild-type animals (Fig. 6A). Null *unc-13(s69)* and loss of function *unc-13(e51)* mutants have much less severe egg-laying defects compared to $\text{G}\alpha_q$ signaling mutants, accumulating ~22 and ~19 eggs (Fig. 6A) compared to >30 eggs (Fig. 1C-I). These results suggest that $\text{G}\alpha_q$ promotes synaptic transmission and egg-laying through an UNC-13 independent pathway. If PMA activation of vulval muscle Ca^{2+} and egg laying were mediated by UNC-13, then mutations that eliminate this protein should reduce that response. However, *unc-13* mutants still laid eggs in

response to PMA (Fig. 6B). Because DAG production may be a conserved biochemical consequence of $G\alpha_q$ signaling through both PLC β and Trio RhoGEF pathways, and both effector pathways have functions in neurons, we hypothesized that serotonin might promote egg laying via modulation of UNC-13 activity. In support of this model, *unc-13* mutants failed to lay eggs in response to serotonin (Fig. 6B). To determine whether serotonin promotes egg laying through Protein Kinase C, we analyzed egg accumulation in animals bearing predicted null mutations in different PKC isoforms (Edwards et al., 2012; Hyde et al., 2011; Okochi et al., 2005; Tabuse, 2002; Tabuse et al., 1989). PKC-1 has previously been shown to promote neuropeptide transmission (Sieburth et al., 2007). While neuropeptides signal to promote egg laying (Avery et al., 1993; Brewer et al., 2019; Jacob and Kaplan, 2003; Kass et al., 2001), PKC-1 (nPKC- ϵ) null mutants showed a grossly normal egg accumulation of 14~17 eggs (Fig. 6C). Animals bearing predicted null mutants of novel and conventional PKCs such as nPKC δ/θ (TPA-1) and cPKC α/β (PKC-2) orthologs also show no significant differences in egg accumulation (Fig. 6C), suggesting that disruption of PKC signaling does not strongly affect egg-laying behavior. Like UNC-13, If PMA activation of vulval muscle Ca^{2+} and egg laying were mediated by PKCs, then mutations that eliminate PKCs, should reduce that response. However, PKC single mutant animals still laid eggs in response to PMA (Fig. 6C). Together, these results support a model where serotonin promotes neurotransmitter release and egg laying via UNC-13, however, there are additional targets of DAG that function in parallel to UNC-13 in neurons and/or downstream of UNC-13 in the vulval muscles to promote egg laying.

Discussion

In this study we explored the cellular specificity of $G\alpha_q$ effector signaling as it regulates egg-laying circuit activity and behavior using molecular genetics, optogenetics, pharmacology, and calcium imaging techniques. We found that $G\alpha_q$ effectors PLC β and Trio RhoGEF differentially act in neurons and muscles to promote synaptic transmission and egg-laying behavior. PLC β signals presynaptically in neurons while Trio RhoGEF signals both presynaptically in neurons and postsynaptically in muscles. Although $G\alpha_q$, PLC β , and Trio RhoGEF mutants all fail to lay eggs in response to serotonin, optogenetic stimulation of HSNs fully rescued egg laying in PLC β but not $G\alpha_q$ or Trio RhoGEF mutants, suggesting hyperactivation of the HSNs may be able to bypass PLC β signaling in some cases. Recent work has shown that the HSNs release NLP-3 neuropeptides which can promote egg laying even in *tph-1* mutants lacking serotonin (Brewer et al., 2019), possibly explaining why optogenetic activation of HSNs may be able to stimulate egg laying in PLC β mutants even when exogenous serotonin cannot. The HSNs are also predicted to release Ach (Gendrel et al., 2016; Pereira et al., 2015), which may signal through nAChR receptors expressed on the vulval muscles (Kim et al., 2001; Waggoner et al., 2000). Because serotonin drives egg laying in animals lacking HSNs but not in animals lacking PLC β , we propose that $G\alpha_q$ and PLC β act in neurons other than HSN to promote ACh release onto the vulval muscles for egg laying. The cholinergic VA, VB, and VC neurons also innervate the vulval muscles alongside the HSNs (Cook et al., 2019; White et al., 1986). Consistent with this model, we have recently shown that blocking VC synaptic transmission reduces egg laying in response to serotonin (Kopchock et al., 2021). Optogenetic stimulation of the VCs (Kopchock et al., 2021) or VA/VB neurons (Kopchock et al., unpublished) stimulates vulval muscle Ca^{2+} activity, but the resulting Ca^{2+} activity is insufficient to drive egg laying behavior (data now shown). Because we find that mutations that increase $G\alpha_q$ signaling have stronger vulval muscle Ca^{2+} transients, we

propose that serotonin and NLP-3 released from HSN potentiates both ACh release from other motor neurons and the electrical excitability and/or contractility of the vulval muscles, converting rhythmic twitch Ca^{2+} transients into stronger egg-laying transients. Previous studies have shown that Trio RhoGEF acts in presynaptic motor neurons to regulate locomotion behavior (Hu et al., 2011; Steven et al., 2005; Williams et al., 2007). Our studies show that transgenic Trio RhoGEF expression in either neurons or muscles alone is insufficient to restore wild-type level of egg-laying behavior, but expression in both is sufficient. These results mirror previously published results for $\text{G}\alpha_q$ (Bastiani et al., 2003), further supporting a model where $\text{PLC}\beta$ and Trio RhoGEF have distinct functions during locomotion and egg-laying behaviors.

How do $\text{G}\alpha_q$, $\text{PLC}\beta$ and Trio-RhoGEF signaling promote egg-laying behavior? Earlier studies have suggested that Rho ortholog RHO-1 in *C. elegans* regulates synaptic activity in a mechanism that involves the G12 family protein GPA-12 (Hiley et al., 2006; Lutz et al., 2005). Activated RHO-1 also directly binds to and inhibits the DGK-1 diacylglycerol kinase expressed in neurons that signals to reduce DAG available to bind effectors (Hiley et al., 2006; McMullan et al., 2006). Our data are consistent with the previous results reporting that $\text{G}\alpha_q$ signaling regulates postsynaptic vulval muscle activity mainly through $\text{G}\alpha_q$ -Trio pathway as $\text{G}\alpha_q$, and Trio mutants show a similarly strong reduction in vulval muscle Ca^{2+} activity. Because muscle activity defects in $\text{G}\alpha_q$ and Trio mutants can be restored by the DAG-mimetic PMA, we suggest that insufficient levels of DAG are responsible for the circuit activity and behavior defects of $\text{G}\alpha_q$ and Trio RhoGEF mutants. In the absence of $\text{PLC}\beta$, how would parallel $\text{G}\alpha_q$ signaling through Trio RhoGEF and RHO-1 generate DAG? Besides $\text{PLC}\beta$ (EGL-8), *C. elegans* expresses four other PLC orthologs: $\text{PLC}\epsilon$ (PLC-1), PLC-2, $\text{PLC}\gamma$ (PLC-3), and $\text{PLC}\delta$ (PLC-4) (Vázquez-Manrique et al., 2008). *In vitro* studies with cultured mammalian cells show that small G proteins like Rho can bind to and activate $\text{PLC}\epsilon$ (Seifert et al., 2008; Wing et al., 2003). Genetic evidence in *C. elegans*

suggests a model that both PLC ϵ and PLC γ likely function downstream of G α_q to promote cell activity (Kunitomo et al., 2013; Yu et al., 2013), but whether Trio activation of Rho ultimately acts through these PLCs to produce DAG is not clear. One approach to test if these other PLCs mediate Rho signaling would be to perform genetic epistasis experiments. However, loss of Rho-1 causes lethality (Jantsch-Plunger et al., 2000; McMullan and Nurrish, 2011) and loss of PLC ϵ and PLC γ cause sterility defects (Yin et al., 2004), limiting our ability to measure differences in egg laying. Imaging or biochemical experiments documenting changes in PIP₂ (Stauffer et al., 1998) and/or DAG (Ohno et al., 2017; Tewson et al., 2012) could allow for direct tests of whether G α_q signaling through Trio and Rho changes PIP₂ and DAG levels *in vivo*.

Previous studies have shown that the DAG mimetic phorbol esters promote both synaptic vesicle and dense core vesicle release from neurons and neurosecretory cells (Silinsky and Searl, 2003). DAG and phorbol esters activate many effectors including mUNC-13 in the brain and Protein Kinase C (PKC) in nearly all cells (Betz et al., 1998; Huang, 1989). In *C. elegans*, treatment of exogenous PMA promotes hypersensitivity to the paralytic effects of aldicarb (Sieburth et al., 2007). Double mutants of both PKC-1 null and UNC-13 (H17K) show a greater magnitude of resistivity to phorbol esters compared to either mutant alone suggesting that PMA acts in part through these effectors to regulate ACh release (Sieburth et al., 2007; Silinsky and Searl, 2003). Our data show that animals lacking either UNC-13 or single PKC isoforms still show a robust egg-laying response to PMA (Fig. 6), suggesting DAG and PMA promote muscle activity via other targets. *In vitro* studies show ROCK (Rho-associated coiled-coil kinase) activation in PMA-induced apoptosis and macrophage differentiation (Chang et al., 2006; Yang et al., 2017). ROCK has a predicted C1 domain that mediates protein interaction with DAG and might bind and be similarly activated by PMA (Xiao et al., 2009). In *C. elegans*, RHO-1 signals through LET-502/ROCK to phosphorylate non-muscle myosin light chain (Shimizu et al., 2018). G α_q also promotes neurotransmitter release via additional kinase targets including SEK-1

Mitogen-Activated Protein Kinase Kinase in the p38 MAPK pathway and KSR-1 in the ERK MAPK pathway (Coleman et al., 2018; Hoyt et al., 2017). KSR-1 is particularly interesting in that its N-terminus shares sequence similarly with C1 domains that might mediate regulation by DAG. Loss of KSR-1 and other ERK MAPK components also suppress the loopy locomotion defects caused by gain-of-function mutations in Rho-1 (Coleman et al., 2018). Taken together, our work is consistent with a model where additional DAG-sensitive effectors act downstream of $G\alpha_q$, Trio and Rho to promote muscle excitability and /or contractility for egg laying.

Apart from direct activation of C1 domain containing effectors, emerging evidence indicates Phospholipase C-dependent production of DAG can regulate neural circuit activity via endocannabinoid-mediated retrograde signaling. In pyramidal cells of the mouse hippocampus, postsynaptic calcium influx through voltage-gated calcium channels activates PLC β 1 to generate DAG (Hashimotodani et al., 2006; Hashimotodani et al., 2007; Soltesz et al., 2015). Diacylglycerol lipase- α (DAGL α) can convert DAG to Arachidonoylglycerol (2-AG), which signals from dendrites in a retrograde manner through presynaptic $G\alpha_o$ -coupled endocannabinoid receptors to inhibit neurotransmitter release (Hashimotodani et al., 2013; Hashimotodani et al., 2005; Soltesz et al., 2015; Tanimura et al., 2010; Wettschureck et al., 2006). In *C. elegans*, 2-AG activates the NPR-19 endocannabinoid receptor ortholog that couples to $G\alpha_o$ to modulate serotonin transmission, pharyngeal, feeding, and locomotory behaviors (Oakes et al., 2019; Oakes et al., 2017; Pastuhov et al., 2016). We have recently shown that feedback of egg accumulation alters vulval muscle Ca^{2+} activity, which subsequently signals to regulate bursts of Ca^{2+} transients in the HSNs that accompany the onset of the egg-laying active state (Ravi et al., 2018a; Ravi et al., 2021). These results support a model where stretch-dependent feedback of egg accumulation stimulates postsynaptic vulval muscle Ca^{2+} signaling. This Ca^{2+} could then activate PLCs to generate DAG and 2-AG which signal to modulate HSN activity, serotonin release, and egg laying. The genetic and experimental accessibility of the *C. elegans* egg-laying

579 circuit should allow us to determine if conserved G proteins like $G\alpha_q$ act generally to drive neural
580 circuit activity via changes in DAG, subsequent activation of effectors, and retrograde
581 messengers like 2-AG.

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Figure 1: Trio RhoGEF is required in both neurons and muscles to regulate egg-laying behavior (A) Schematics of excitatory (black) and inhibitory (red) $G\alpha_q$ signaling pathway. *C. elegans* gene names are beneath the protein they encode. (B) Cartoon of the *C. elegans* egg-laying behavior circuit from a lateral view. Only the left side of the bilaterally symmetric circuit is shown. HSNL, Hermaphrodite Specific Neuron (left); VC4 and VC5 Ventral C neurons; vm1 and vm2 vulval muscles, um1 and um2 uterine muscles; uv1 uterine-vulval neuroendocrine cells. (C-H) Bright field images of worms of the indicated genotypes; arrowheads indicate accumulated eggs. Mean number of accumulated eggs $\pm$ 95% confidence intervals is also indicated. Position of the vulva is shown with asterisk (*). (I) Scatterplot of egg accumulation in wild-type, *eat-16(tm761)*, *egl-30(tg26)*, *egl-30(ad805)*, *egl-8(sa47)*, and *unc-73(ce362)* mutant animals. Line indicates mean eggs $\pm$ 95% confidence intervals. Asterisks indicate $p \leq 0.0001$ (One way ANOVA with Bonferroni's correction; wild type (n=49); *eat-16(tm761)* (n=36); *egl-30(tg26)* (n=47); *egl-30(ad805)* (n=44); *egl-8(sa47)* (n=65); *unc-73(ce362)* (n=38). (J) Transgenic rescue of *egl-8* PLC β egg-laying defects. Scatterplot of egg accumulation in transgenic animals expressing GFP only or EGL-8/PLC β fused to GFP from the *rgs-1* promoter in *egl-8(sa47)* null mutants (n=50) compared to wild-type (n=30) and *egl-8(sa47)* mutant animals (n=30). Bar indicates mean eggs $\pm$ 95% confidence intervals. Asterisks indicate $p \leq 0.0001$ (One-way ANOVA with Bonferroni's correction) (K) Transgenic rescue of *unc-73* Trio RhoGEF egg-laying defects. Scatterplot of egg accumulation in wild-type (n=60), *unc-73(ce362)* mutants (n=72), and transgenic animals expressing a fluorescent protein with or without Trio in neurons (n $\geq$ 32) or in muscles (n $\geq$ 69), or in both neurons and muscles (n $\geq$ 41) in *unc-73(ce362)* mutants. Horizontal line indicates mean accumulated eggs $\pm$ 95% confidence intervals. Asterisks indicate $p \leq 0.0145$; n.s.= not significant ($p > 0.05$; One-way ANOVA with Bonferroni's correction for multiple comparisons).

616

617 **Figure 2: Serotonin signals through $G\alpha_q$, Trio, and $PLC\beta$ to promote egg-laying.**

618 **(A)** Model of serotonergic and acetylcholine (ACh) signaling in the egg-laying circuit. **(B)** Bar
619 plots showing the percentage of animals laying eggs in M9 buffer alone (grey) or M9 +18.5 mM
620 serotonin (green) in 1 hr. Bar indicates mean percent $\pm$ 95% confidence intervals. Asterisks
621 indicate p<0.0007; n.s.= not significant (p>0.05, Fisher's exact test with Bonferroni's correction
622 for multiple comparisons; n>30 animals). **(C)** Bar plot showing percent of animals laying eggs in
623 M9 buffer or M9 +18.5 mM serotonin in wild-type or Trio RhoGEF mutant animals expressing
624 nothing or Trio RhoGEF in neurons, muscles, or both. Bar indicates mean percent $\pm$ 95%
625 confidence intervals. Asterisks indicate p<0.0007; n.s.= not significant (p>0.05, Fisher's exact
626 test with Bonferroni's correction for multiple comparisons; n>30 animals).

Figure 3: Optogenetic stimulation of the HSNs or vulval muscles indicates cellular specificity of $G\alpha_q$ effectors for egg laying. (A) On left, cartoon of the egg-laying circuit and experiment showing blue light activation of HSN for 30 seconds. On right, scatterplot showing eggs laid per worm in presence (+) or absence (-) of all-*trans* retinal (ATR) cofactor during the blue light activation of ChR2 expressed in HSNs of wild-type (grey), *egl-30(ad805)* $G\alpha_q$ strong loss-of-function mutants (red), *egl-8(n488)* and *egl-8(sa47)* PLC β mutants (orange), and *unc-73(ce362)* Trio mutant (purple) animals. Line indicates mean eggs laid $\pm 95\%$ confidence

636 intervals. Asterisks indicate $p < 0.0001$; n.s.= not significant ($p > 0.05$, One-way ANOVA with
637 Bonferroni's correction for multiple comparisons; n= how many animals?). **(B)** On the left,
638 cartoon of the egg-laying circuit and experiment showing blue light activation of vulval muscles
639 for 30 seconds(left). On the right, scatter plots of eggs laid per worm in presence (+) or absence
640 (-) of ATR during blue light activation of ChR2 expressed in the vulval muscles of wild type, *egl-*
641 *30(ad805)* $G\alpha_q$ strong loss-of-function mutants (red), *egl-8(n488)* and *egl-8(sa47)* PLC β mutants
642 (orange), and *unc-73(ce362)* Trio mutant (purple) animals. Line indicates mean eggs laid $\pm 95\%$
643 confidence intervals. Asterisk indicates $p \leq 0.0255$; n.s.=not significant, $p > 0.05$ (One-way ANOVA
644 with Bonferroni's correction for multiple comparisons; $n > 10$).

Figure 4: $\text{G}\alpha_q$ and Trio signaling promotes vulval muscle activity. (A) Representative GCaMP5::mCherry ($\Delta\text{R/R}$) ratio traces showing vulval muscle Ca^{2+} activity in freely behaving wild-type (black), *egl-30(n686)* $\text{G}\alpha_q$ weak loss of function mutant (red), *egl-30(ad805)* $\text{G}\alpha_q$ strong loss-of-function mutant (red), *unc-73(ce362)* Trio strong loss-of-function mutant (purple), *egl-8(n488)* PLC β null mutant (light blue), *egl-8(sa47)* PLC β null mutant (orange), *eat-16(tm761)* $\text{G}\alpha_q$ RGS protein null mutant (grey), *egl-30(tg26)* strong $\text{G}\alpha_q$ gain-of-function mutant (magenta), and *dgk-1(nu62)* DAG Kinase null mutant (dark blue) animals during active (green solid bar) and

653 inactive (dotted grey line) egg-laying behavior states. Arrowheads indicate egg-laying events.
654 Vertical and horizontal scale bars show GCaMP5/mCherry fluorescence ratio ($\Delta R/R$) and time,
655 respectively **(B)** Scatterplots of Ca^{2+} transient frequency for indicated genotypes. Line indicates
656 mean eggs laid $\pm 95\%$ confidence intervals; asterisks indicate $p \leq 0.0340$; n.s. indicates not
657 significant ($p > 0.05$, One-way ANOVA with Bonferroni's correction for multiple comparisons;
658 $n > 10$ animals recorded per genotype). **(C)** Scatterplots of Ca^{2+} peak amplitudes for the indicated
659 genotypes during twitch (closed square) and egg-laying transients (open circles). Asterisks
660 indicate $p < 0.0001$, n.s. indicates not significant ($p > 0.05$, Kruskal Wallis test with Dunn's
661 correction for multiple comparisons).

Figure 5: DAG mimetic PMA rescues egg-laying circuit activity and behavior defects of $G\alpha_q$ signaling mutants. **(A)** Diagrams showing working model of $G\alpha_q$ and DAG signaling pathway during egg-laying behavior. **(B)** Bar plots showing the percentage of animals showing egg laying in M9 buffer (open grey bars) or M9 buffer +10 μ M PMA (filled green bars) Sample size for each genotype and each condition. Error bars indicate 95% confidence intervals for the proportion; asterisks indicate $p < 0.0006$ (Fisher's exact test with Bonferroni's correction for multiple comparisons; $n \geq 30$ animals per genotype and condition). **(C)** Left, representative GCaMP5::mCherry ($\Delta R/R$) ratio traces showing vulval muscle Ca^{2+} activity in wild-type or the indicated $G\alpha_q$ signaling mutant animals in the absence or presence of 10 μ M PMA. Arrowheads indicate egg-laying events. Vertical and horizontal scale bars show GCaMP5/mCherry fluorescence ratio ($\Delta R/R$) and time, respectively. Right, heat map showing intensity modulated color spectrum of GCaMP5::mCherry ($\Delta R/R$) ratio of vulval muscle Ca^{2+} activity ranging from blue (low Ca^{2+}) to red (high Ca^{2+}) of 10 animals. **(D)** Scatterplots of Ca^{2+} transient frequency in the absence (-) and presence (+) of 10 μ M PMA for the indicated genotypes. Line indicates mean eggs laid $\pm$ 95% confidence intervals; asterisk indicates $p \leq 0.0275$; n.s.= not significant ($p > 0.05$, One-way ANOVA with Bonferroni's correction for multiple comparisons; $n \geq 10$ animals per genotype and condition).

Figure 6: DAG promotes egg laying independent of UNC-13 or Protein Kinase C. (A)

Scatterplot of egg accumulation in wild-type, *unc-13(e51)* loss-of-function mutant, and *unc-13(s69)* null mutant animals. Line indicates mean eggs laid $\pm$ 95% confidence intervals. Asterisk indicates $p \leq 0.0021$ (One-way ANOVA with Bonferroni's correction for multiple comparisons; $n \geq 36$ per genotype and condition). **(B)** Bar plots showing the percentage of wild-type, *unc-13(e51)*, or *unc-13(s69)* mutant animals laying eggs in M9 buffer (grey open boxes), 18.5 mM serotonin (filled blue boxes), or 10 μ M PMA (filled green boxes). Bar indicates mean eggs $\pm$ 95% confidence interval for the proportion. Asterisks indicate $p < 0.0006$; n. s = not significant ($p > 0.05$, Fisher's exact test with Bonferroni's correction for multiple comparisons; $n \geq 36$ animals per genotype and condition). **(C)** Scatterplot of egg accumulation in wild-type ($n=24$) and indicated Protein Kinase C mutant animals ($n \geq 35$ per genotype). Line indicates average eggs accumulated

693 $\pm 95\%$ confidence intervals. n.s.= not significant ($p > 0.05$, One-way ANOVA with Bonferroni's
694 correction for multiple comparisons **(D)** Bar plots showing the percentage of wild-type and
695 Protein Kinase C mutant animals showing egg laying in M9 buffer (open bars) or 10 μM PMA
696 (filled green bars). Bar indicates mean eggs $\pm 95\%$ confidence intervals for the proportion.
697 Asterisks indicate $p < 0.0007$; n.s.= not significant ($p > 0.05$, Fisher's exact test with Bonferroni
698 correction for multiple comparisons; $n \geq 35$ animals per genotype and condition).

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Movie 1: GCaMP5:mCherry fluorescence ratio in the vulval muscles of wild-type animals in the absence or presence of 10 μ M DAG analogue, PMA. Blue indicates low Ca^{2+} , and red indicates elevated Ca^{2+} .

Movie 2: GCaMP5:mCherry fluorescence ratio in the vulval muscles of strong loss of function $\text{G}\alpha_q$ mutant, *egl-30(ad805)*, in the absence or presence of 10 μ M DAG analogue, PMA. Blue indicates low Ca^{2+} , and red indicates elevated Ca^{2+} .

Movie 3: GCaMP5:mCherry fluorescence ratio in the vulval muscles of strong loss of function Trio mutant, *unc-73(ce362)*, animals in the absence or presence of PMA. Blue indicates low Ca^{2+} , and red indicates elevated Ca^{2+} .

Table 1: Lists of *C. elegans* strains used in this study

Strains	Genotype	Feature	Figure(s)	Source
N2	Wild type	Bristol wild-type strain	1,2,5	(Brenner, 1974)
MT1434	<i>egl-30(n686) I</i>	$\text{G}\alpha_q$ loss-of-function mutant, Egg-laying defective	1,2,5	(Trent et al., 1983)
DA823	<i>egl-30(ad805) I</i>	$\text{G}\alpha_q$ strong loss-of-function mutant, Egg-laying defective	1,2,5	(Brundage et al., 1996; Mendel et al., 1995)
KG1278	<i>unc-73(ce362) I</i>	Trio RhoGEF loss-of-function mutant, Egg-laying defective	1,2	(Williams et al., 2007)

KG1397	<i>unc-73(ev802) I</i>	Trio RhoGEF deletion mutant, Egg-laying defective	2,5	(Williams et al., 2007)
JT47	<i>egl-8(sa47) V</i>	PLC β null, Egg-laying defective	1,2,5	(Thomas, 1990)
MT1083	<i>egl-8(n488) V</i>	PLC β null, Egg-laying defective	2	(Trent et al., 1983)
LX1226	<i>eat-16(tm761) I</i>	G α_q RGS null, Hyperactive egg laying	1	(Porter and Koelle, 2010)
CG21	<i>egl-30(tg26) I; him-5(e1490) V</i>	G α_q gain-of-function mutant, Hyperactive egg-laying	1	(Garcia et al., 2001)
LX1832	<i>lite-1(ce314) lin-15(n765ts) X</i>	strain used for transgenic line creation		(Gürel et al., 2012)
LX1286	<i>egl-8(sa47) I; lin-15(n765ts) X</i>	PLC β null mutant, Egg-laying defective; multi-vulva	1	This study
LX1674	<i>egl-8(sa47) I; lin-15(n765ts) X; vsEx679</i>	PLC β null, Egg-laying defective; non-Muv, expresses GFP in neurons from <i>rgs-1</i> promoter	1	This study
LX1675	<i>egl-8(sa47) I; lin-15(n765ts) X; vsEx680</i>	PLC β null, Egg laying defective; non-Muv, expresses PLC β and GFP in neurons from <i>rgs-1</i> promoter	1	This study
MIA26	<i>egl-1(n986dm) V</i>	Lacks HSNs	2,5	(Ravi et al., 2018a)
LX1918	<i>vsIs164 lite-1(ce314) lin-15(n765ts) X</i>	Expresses GCaMP5 and mCherry in the vulval muscles	4,5	(Li et al., 2013)

MIA109	<i>egl-8(sa47) V;</i> <i>vsIs164 lite-</i> <i>1(ce314), lin-</i> <i>15(n765ts) X</i>	Expresses GCaMP5 and mCherry in the vulval muscles of <i>egl-8(sa47)</i> mutant	4,5	This study
MIA139	<i>egl-30(n686) I;</i> <i>vsIs164 lite-</i> <i>1(ce314) lin-</i> <i>15(n765ts) X</i>	Expresses GCaMP5 and mCherry in the vulval muscles of <i>egl-30(n686)</i>	4,5	This study
MIA140	<i>egl-30(ad805) I;</i> <i>vsIs164 lite-</i> <i>1(ce314), lin-</i> <i>15(n765ts) X</i>	Expresses GCaMP5 and mCherry in the vulval muscles of <i>egl-30(ad805)</i>	4,5	This study
MIA141	<i>unc-73(ce362) I;</i> <i>vsIs164, lite-</i> <i>1(ce314) lin-</i> <i>15(n765ts) X</i>	Expresses GCaMP5 and mCherry in the vulval muscles of <i>unc-73(ce362)</i>	4,5	This study
MIA286	<i>egl-30(tg26) I;</i> <i>vsIs164, lite-</i> <i>1(ce314) lin-</i> <i>15(n765ts) X</i>	Expresses GCaMP5 and mCherry in the vulval muscles of <i>egl-30(tg26)</i>	4	This study
MIA287	<i>eat-16(tm761) I;</i> <i>vsIs164 lite-</i> <i>1(ce314) lin-</i> <i>15(n765ts) X</i>	Expresses GCaMP5 and mCherry in the vulval muscles of <i>eat-16(tm761)</i>	4	This study

MIA288	<i>egl-8(n488) V;</i> <i>vsIs164 lite-1</i> <i>(ce314) lin-</i> <i>15(n765ts) X</i>	Expresses GCaMP5 and mCherry in the vulval muscles of <i>egl-8(n488)</i>	4,5	This study
MIA296	<i>dgk-1(nu62) lite-1(ce314) lin-</i> <i>15(n765ts) X</i>	Expresses GCaMP5 and mCherry in the vulval muscles of <i>dgk-1(nu62)</i>	4	This study
MIA372	<i>unc-73(ce362) I;</i> <i>keyEx64</i>	Expresses GFP in the neurons and mCherry in the muscles of <i>unc-73(ce362)</i>	1	This study
MIA373	<i>unc-73(ce362) I;</i> <i>keyEx65</i>	Expresses GFP and Trio RhoGEF-E cDNA in the neurons, and mCherry and Trio RhoGEF-E cDNA in the muscles of <i>unc-73(ce362)</i>	1	This study
MIA374	<i>unc-73(ce362) I;</i> <i>keyEx66</i>	Expresses GFP in neurons	1	This study
MIA375	<i>unc-73(ce362) I;</i> <i>keyEx67</i>	Expresses GFP and Trio RhoGEF-E cDNA in neurons	1	This study
MIA376	<i>unc-73(ce362) I;</i> <i>keyEx68</i>	Expresses mCherry in muscles	1,2	This study
MIA377	<i>unc-73(ce362) I;</i> <i>keyEx69</i>	Expresses mCherry and Trio RhoGEF-E cDNA in muscles	1,2	This study

LX1836	<i>wzls30 IV; lite-1(ce314) lin-15(n765ts) X</i>	Expresses Channelrhodopsin-2 (ChR2) in HSNs from the <i>egl-6</i> promoter	3	(Collins et al., 2016)
MIA229	<i>keyls48; lite-1(ce314) lin-15(n765ts) X</i>	Expresses ChR2 in vulval muscles from the <i>ceh-24</i> promoter	3	(Kopchock et al., 2021)
MIA300	<i>egl-30(ad805) I; wzls30 IV; lite-1(ce314) X</i>	Expresses ChR2 in HSN of <i>egl-30(ad805)</i> mutant	3	This study
MIA301	<i>egl-30(ad805) I; keyls48; lite-1(ce314) X</i>	Expresses ChR2 in vulval muscles of <i>egl-30(ad805)</i> mutant	3	This study
MIA304	<i>wzls30 IV; egl-8(n488) V; lite-1(ce314) X</i>	Expresses ChR2 in HSNs of <i>egl-8(n488)</i> mutant	3	This study
MIA305	<i>egl-8(n488) V; keyls48; lite-1(ce314) X</i>	Expresses ChR2 in vulval muscles of <i>egl-8(n488)</i> mutant	3	This study
MIA308	<i>wzls30 IV; egl-8(sa47) V; lite-1(ce314) X</i>	Express ChR2 in HSNs of <i>egl-8(sa47)</i> mutant	3	This study
MIA309	<i>egl-8(sa47) V; keyls48; lite-1(ce314) X</i>	Expresses ChR2 in vulval muscles of <i>egl-8(sa47)</i> mutant	3	This study

MIA247	<i>unc-73(ce362) I; wzls30 IV; lite-1(ce314) X</i>	Expresses Chr2 in HSN of <i>unc-73(ce362)</i> mutant	3	This study
MIA248	<i>unc-73(ce362) I; keyls48; lite-1(ce314) X</i>	Expresses Chr2 in vulval muscles of <i>unc-73(ce362)</i> mutant	3	This study
MT7929	<i>unc-13(e51) I</i>	UNC-13 loss-of-function	6	(Brenner, 1974)
EG9631	<i>unc-13(s69) I</i>	UNC-13 null	6	(Rose and Baillie, 1980)
IK105	<i>pkc-1(nj1) V</i>	nPKC ϵ null	6	(Okochi et al., 2005)
IK130	<i>pkc-1(nj3) V</i>	nPKC ϵ null	6	(Okochi et al., 2005)
RB781	<i>pkc-1(ok563) V</i>	nPKC ϵ null	6	(Consortium, 2012)
VC127	<i>pkc-2(ok328) X</i>	cPKC α/β null	6	(Consortium, 2012)
MJ500	<i>tpa-1(k501) IV</i>	nPKC δ/θ null	6	(Tabuse et al., 1989)
MJ563	<i>tpa-1(k530) IV</i>	nPKC δ/θ null	6	(Tabuse et al., 1989)

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716 **Table 2: Lists of oligonucleotide sequences used in this study**

Oligo Name	Sequence (5'>3')	Use
lite-1(ce314)-fwd	ACGGGAGACGAAGAGCTAAAT AGG	Genotyping of <i>lite-1(ce314)</i>
lite-1(ce314)-rev	CTAAGTTGCCGGTTGCCTTAG AAC	Genotyping of <i>lite-1(ce314)</i>
egl-30(n686)-F	GCCAACCGAGCAGGACATTCTG CG	Genotyping of <i>egl-30(n686)</i>
egl-30(n686)-R	CGGGAAAGTAGTCAGCGAGAT GCG	Genotyping of <i>egl-30(n686)</i>
egl-30(ad805)-F	GCCAGGGCTGTCCCATTACGG	Genotyping of <i>egl-30(ad805)</i>
egl-30(ad805)-R	TCGGAAAGCGCCACCAGGAAC	Genotyping of <i>egl-30(ad805)</i>
egl-8-cDNA-fwd	CTTGGCTAGCGTAGAAAAAATG GCAAAGGAGTTCCAGTTC	For amplification of <i>egl-8</i> coding sequences
egl-8-cDNA-rev	CGCCCATGGTTATCAAACGACA GAAGTCGGTTGAGC	For amplification of <i>egl-8</i> coding sequences
egl-8-Cterm-NotI-fwd	GTGGGTACTCCACTGGGGGTG CGGCCGCTGGAGGTCCTTCGA CACCGGT	Quick change mutagenesis of <i>egl-8</i> cDNA to insert in-frame NotI enzyme for GFP insertion
egl-8-Cterm-NotI-rev	ACCGGTGTCTGAAGGACCTCCA GCGGCCGCACCC CCAGTGGAGTACCCAC	Quick-change mutagenesis of <i>egl-8</i> cDNA to insert in-frame NotI enzyme for GFP insertion

NotI-GFP-FWD	GGTGCGGCCGCTGGAAGTAAA GGAGAAGAACTTTTC	For amplification of GFP with flanking, in-frame NotI enzyme sites for insertion into <i>egl-8</i> cDNA
NotI-GFP-REV	TCCAGCGGCCGCTCCTTTGTAT AGTTCATCCATGCC	For amplification of GFP with flanking, in-frame NotI enzyme sites for insertion into <i>egl-8</i> cDNA
RE-GFP-FWD-new	GCGTCTAGAACCGGTGCTAGC GTAGAAAAAATGGTCAGTAAAG GAGAAGAACTTTTC	For amplification of sequences encoding GFP for cloning into pGP3 <i>rgs-1</i> promoter containing plasmid
RE-GFP-REV	TACGAATTCGGTACCTCAGATT TATTTGTATAGTTCATCCATG	For amplification of sequences encoding GFP for cloning into pGP3 <i>rgs-1</i> promoter containing plasmid

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